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(54) Title: METHOD AND DEVICE FOR IDENTIFYING A MYCOBACTERIUM SPECIES RESPONSIBLE FOR A MYCOBACTERIAL INFECTION

(57) Abstract

The invention relates to a method for identifying a *Mycobacterium* species responsible for a mycobacterial infection in human or animal, comprising selecting a suitable mycobacterial species and strain: preparing at least one mycobacterial antigen, respectively antigen preparation: binding the antigen, respectively the antigen preparation to a suitable carrier: causing the binding antigen to react with antibodies from serum of an individual infected with a *Mycobacterium* species: making visible antigen-antibody reactions for a suitable antibody (sub-)class: and identifying the responsible *Mycobacterium* species on the basis of the reactions which are made visible. The invention further provides a diagnostic kit which takes the form of a dip-stick on which is arranged a carrier strip with mycobacterial antigens binding thereto, and means for visualizing antigen-antibody reactions occurring on the carrier after contact with the serum for testing. In another embodiment the diagnostic kit comprises a microtiter plate, in the wells of which a specified antibody is arranged, and means for making visible antigen-antibody reactions occurring in the wells after contact with the serum for testing. The third embodiment is an immunoblot with mycobacterial antigens separated by electrophoresis binding thereto, and means for visualizing antigen-antibody reactions occurring on the immunoblot after contact with the serum for testing.
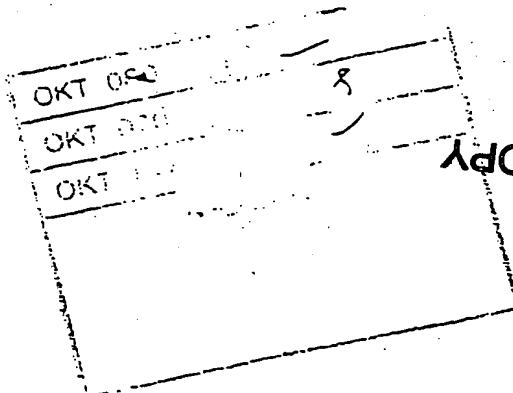
METHOD AND DEVICE FOR IDENTIFYING A MYCOBACTERIUM SPECIES RESPONSIBLE FOR A MYCOBACTERIAL INFECTION

The invention relates to a method for identifying a _Mycobacterium_ species responsible for a mycobacterial infection in human or animal. The invention further relates to diagnostic kits for use in the method.

5   The genus _Mycobacterium_ which contains about 50 species is responsible for a number of human and animal diseases which are known collectively as the mycobacterioses. The best known of these in humans is leprosy, caused by M. leprae, which affects more than ten million people

10 worldwide, and tuberculosis, usually caused by M. tuberculosis, at least ten million new cases of which occur each year. Most other mycobacteria normally occur only as environmental saprophytes but can also cause opportunist diseases. This happens usually, but not only, in the case of

15 people who have problems with their immune system, such as AIDS patients and people undergoing immunosuppression. These opportunist types comprise the slow-growing species M. avium, and the closely related M. intracellulare and M. scrofulaceum (often referred to together as MAIS complex),

20 M. kansasi, M. marinum and M. ulcerans, and the fast-growing species M. chelonae and M. fortuitum. Although once rare, the incidence of opportunist mycobacterial diseases and tuberculosis shows a parallel increase in the western world with the incidence of AIDS. In addition there is limited but

25 increasing evidence that mycobacteria or antigens thereof play a direct or indirect part in the etiology of a plurality of other diseases such as sarcoidosis and Crohn's disease and different auto-immune diseases such as auto-immune dermatitis, rheumatoid arthritis and diabetes. This

30 could be attributed to a structural mimicry between epitopes of mycobacteria and those of the host.

The cell walls of mycobacteria are very complex and contain many lipids, some with structures unique to the

genus. These structures comprise mycolinic acids and esters,
peptido-glycolipide, arabino-galactane and lipo-arabino-
manane. The lipid-rich mycobacterial cell walls are respon-
sible for the characterizing colouring properties of the
5 mycobacteria. They also enable mycobacteria to counter an
attack by the immune system of the host. A number of spe-
cies, once taken up into macrophages, are capable of sur-
rounding themselves with a thick layer of secreted lipids.

Many different components of the mycobacteria begin an
10 interaction with the immune system. These components compri-
se protein and hydrocarbon antigens, which can either be
actively secreted by the mycobacteria or can form part of
the cell wall or cell membrane. In addition they may be
present in the cytoplasm, for instance in the cytoplasmic
15 matrix, ribosomes and enzymes. Mycobacteria also possess
immuno-modulating components such as immunosuppressing
compounds and adjuvants. Consequently, a single mycobacteri-
al species can induce a large variety of immune responses in
different forms and with diverse specificities. It is there-
20 fore difficult to distinguish immune responses against
species-specific components from cross reactions. For this
reason it has therefore been found difficult to derive
protein antigens suitable for the detection of species-
specific humoral responses as a basis for a very sensitive
25 and specific sero-diagnostic test for tuberculosis. Because
the mycobacteria occur a great deal in the environment,
human serum nearly always contains anti-mycobacterial anti-
bodies.

In view of the problems with the specificity of protein
30 antigens, a number of researchers, including the present
inventors, have focused their attention on species-specific
glycolipid antigens for the detection of specific humoral
immune responses. This approach is for example illustrated
in Vega-Lopez et al., J. Clin. Microbiol. 26(12), 2474-2479
35 (1988) and Roche et al., Int. J. Mycobact. Dis. 60(2), 201-
207 (1992) who both stated that in serodiagnosis species-
specific antigens and antibodies are required. Although the
immune reactivity against mycobacteria is of the cell-media-

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ted type and the humoral immune responses probably play a
minor part in the total effector mechanism of mycobacterial
5 immunity and immunopathology, studies into the antibody
response to

immuno-dominant mycobacterial cross-reactive antigen
components (further referred to as Im-CRAC) could shed light
on the varying capability of the host to recognize different
mycobacterial antigens. They could therefore provide
5   indirect information relating to the nature of the immune
recognition of, and response to, a specific mycobacterial
pathogen.

It has now been found that the clinical manifestation
of mycobacterial diseases appears to be related to the
10 varying capability of an individual host to produce a
humoral response to different mycobacterial immuno-cross-
reactive antigen components (Im-CRAC). Each mycobacterial
infection generates its own specific antibody response to a
number of specified antigens. Analysis of the antibody-
15 response by means of immunoblotting has demonstrated that
the immuno-dominant Im-CRAC vary in accordance with the
immunopathological manifestation of the mycobacterial
diseases. It has been found that the sera of individuals
which are infected with different _Mycobacterium_ species
20 cause different and distinguishing band patterns on
immunoblots of mycobacterial antigens.

This discovery forms the basis of the present inven-
tion, whereby a method is provided for identifying a
Mycobacterium species responsible for a mycobacterial
25 infection in human or animal, comprising the steps of:

a) selecting a suitable mycobacterial species and
strain;

b) preparing at least one mycobacterial antigen,
respectively antigen preparation;

30   c) binding the antigen, respectively the antigen
preparation to a suitable carrier;

d) causing the binding antigen to react with antibodies
from serum of an individual infected with a _Mycobacterium_
species;

35   e) making visible antigen-antibody reactions for a
suitable antibody (sub-)class; and

f) identifying the responsible _Mycobacterium_ species on
the basis of the reactions which are made visible.

In preference the antigen preparation is separated by electrophoresis prior to step c) and the carrier is a membrane to which the antigen is bound by means of electroblotting. This process is called Western blotting.

5   The Im-CRAC comprise namely a number of antigens with specific molecular weights which, as has now been found, after immunoblotting exhibit a binding pattern which correlates to the disease, respectively infection. The specific band pattern is characterized by the presence or

10 absence of four individual components, for instance:

-       a region comprising different pronounced bands and/ or overlapping bands, which can be observed as a smear ("region");

-       sharp single bands which are strongly positive

15   ("band");

-       sharp double bands which are strongly positive ("doublet"); and

-       other positive bands ("extra bands").

For a survey of the different antigens, their molecular

20 weights and binding characteristics, see table 1.

Table 1:

Survey of characteristic binding patterns of mycobacterial Immuno-Cross-reactive Antigen Components.

Antigen	Diagnostic for	MW range (in KDa)	Binding characteristic
A	J	< 8	band
B	T, B, J,	10-16	band
C	B, J, T,	20-28	band or region
D	L	29-33	doublet
E	B, J, T,	31-40	band or region
F	T	38-40	band or region
G	C, B, J,	45-48	doublet
H	T	58-60	band or region
I	L	64-65	doublet
J	J	66	band
K	T	68	band
L	L	30-64	region

T: Human Tuberculosis
L: Leprosy
C: Crohn's Disease
B: Bovine Tuberculosis
J: Johne's Disease

The mycobacterioses are all characterized by a specific banding pattern which is formed when a blot having thereon an antigen preparation of mycobacteria separated to size is incubated with serum of an infected individual.

The tables 2a-2e below show a survey of the banding patterns of a number of mycobacterial, respectively immunological diseases.

Table 2a:

Bovine tuberculosis

Regions (MW in KDa)

Pattern	and/or 10-16	and/or 20-28	and/or 31-40	and 45-48
1.	14 KDa band	22 KDa band 20-28 KDa region	31 KDa band	45/48 KDa doublet

Johne's Disease

Regions (MW in KDa)

Pattern	and/or > 8	and 10-16	and/or 20-28	and/or 31-40	and 45-48	and 66
1.	region	14 KDa band	and 22 (25)-KDa band, and/or 27 KDa band	31 KDa band	45/48 KDa doublet	66 KDa band

Table 2b:

Human Tuberculosis

Regions (MW in KDa)

Pattern	10-16	20-28	31-38	38-40	58-60	68	other*
1.	10 and 16 KDa bands	/	33 KDa band	region	bands	/	+/-
2.	16 KDa single band	region	33 KDa band	bands	region	/	+/-
3.	10 or 16 KDa band	/	/	bands	bands	/	+/-
4.	16 KDa band	/	33 KDa band	region	bands	/	+/-
5.	10 and/or 16 KDa band	region	33 KDa band	bands	bands and/or region	68 KDa band	+/-

* Extra bands and/or regions can colour but are not diagnostic for Human Tuberculosis

Table 2c:

Leprosy

Regions (MW in KDa)

Pattern	29-33	30-65	64-65	other*
LL pattern 1	29/33 KDa doublet	/	/	+/-
TT pattern 1	/	regions and/or bands	/	+/-
TT pattern 2	/	regions and/or bands	64/65 KDa doublet	+/-
TT pattern 3	/	/	64/65 KDa doublet	+/-

* Extra bands and/or regions can colour but are not diagnostic for leprosy

Table 2d:

Crohn's Disease

Regions (MW in KDa)

Pattern	45-48	other*
1.	45/48 KDa doublet	+/-

* Extra bands and/or regions can colour but are not diagnostic for Crohn's Disease

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Table 2e:

Regions (MW in KDa)

Pattern	and 42 KDa	and/or 80-90 KDa	and/or 58-60 KDa	and/or 14-18 KDa
1.	band	region	region	region

When an immunoblot is used, two questions can be
answered. Firstly, the presence of any positive band pattern
will answer the question of whether a mycobacterial
infection is present. Secondly, the presence of specific
banding patterns indicates which mycobacterial species has
caused the infection, and therefore what the nature and
etiology of the disease will be.

The invention further relates to a heterogeneous enzyme
immunoassay.

From the patterns in the immunoblotting it follows
which mycobacterial antigens respectively antigen prepara-
tions are suitable for diagnosis of any particular disease.

The antigen for a heterogeneous enzyme immunoassay is
preferably chosen from the group which consists of myco-
bacterial immuno-cross-reactive antigen components with a
molecular weight of 29-33 KDa, 45-48 KDa, 64-65 KDa and a
fraction designated with the term KP-100. These ImCRAC can
be used separately or in combination with each other for
serological diagnosis of the correlating diseases in a
heterogeneous enzyme immunoassay (EIA).

In this form of assay antibody-conjugates labelled with
a standard enzyme are used. An important detail is that the
enzyme activity does <u>not</u> change during the immunological
reaction.

In order to test the immune response in patients to the
selected antigens, use is made for instance of microtiter
plates ("Solid Phase"). By means of standard published

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techniques the antigens are irreversibly immobilized on the
surface of the wells in such a microtiter plate.

    This binding takes place while retaining specific
antigen determinants on the used antigens. After incubation
5 with serum, in the wells of the microtiter plate, antibodies
present therein can specifically form a complex with the
irreversibly bound antigens.

    After removal of non-binding serum components, binding
antibodies are detected using an anti-antibody antibody
10 labelled with an enzyme.

    Binding of the enzyme is only possible when specific
antibodies have adhered to the immobilized antigens.
Substrate conversion by the binding enzyme to a visually or
photometrically observable signal is thereby directly
15 related to the presence of specific antibodies in the tested
serum.

    The choice of specificity of the enzyme-bound anti-
antibody antibody determines the type of reaction that takes
place. For instance, it may be desirable in some cases to
20 demonstrate the specifically binding immunoglobulins of the
IgG type, while in other cases immunoglobulins of the IgA
and/or IgM type are just demonstrated.

    The combination of antigen and immunoglobulin type
defines the specificity of the test.

25   The said methods, that is, the immunoblot and the EIA,
can be used as mutual confirmation.

    In addition, for the serological diagnosis based on the
said antigens, use can be made of a test stick as solid
phase.

30   A particularly advantageous embodiment of the invention
relates to a test stick, the so-called "dip-stick", which is
used as solid phase in the heterogeneous enzyme immunoassay.

    The said mycobacterial antigens can be irreversibly
bound to such a dip-stick.

35   The antigen is brought into reaction with antibody from
serum for testing by dipping the dip-stick in a serum sample
for testing. The formed antigen-antibody complex can be made

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visible by subsequently dipping the dip-stick in an anti-
antibody antibody-enzyme conjugate solution.

    With the binding enzyme a substrate can then be
converted to a visually or photometrically observable
5 signal.

    The invention provides a diagnostic kit for:
    - an immunoblot assay; comprising ImCRAC antigens
separated by electrophoresis as described above, immobilized
on a solid carrier, in addition to an associated suitable
10 detection system.

    - a heterogeneous enzyme immunological assay; com-
prising a microtiter plate, the wells of which are coated
with above mentioned antigens or antigen preparations, in
addition to an associated suitable detection system.

15   - a dip-stick assay; comprising test sticks coated with
antigen or antigen preparations, in addition to an
associated suitable detection system.

    The present invention will be further elucidated with
reference to a number of examples which are given herein by
20 way of illustration and are not intended to limit the
invention.

EXAMPLE 1 Immunoblot

1.   Preparation of crude mycobacterial mass ("starting
25   material").

    The mycobacteria were cultured in commercially avail-
able Sauton medium supplemented with 2 g $MgSO_4$, 8 g citric
acid, 2g K_2HPO_4, 16 g asparagine, 2 g (Fe^+) ammonium citrate,
240 ml glycerol. The bacteria were cultured under standard
30 conditions. The cells were harvested by filtration of the
culture medium with a 12 μm filter. The cells were
subsequently resuspended in 20 ml PBS (phosphate-buffered
salt solution) (pH 7.4) and the harvested cells were
autoclaved under a pressure of 15 Psi for 20 minutes in
35 order to deactivate and sterilize the bacteria. The thus
obtained bacterial mass can be stored at -80°C.

    In order to determine the quantity of starting material
a 1/100 dilution of the harvested autoclaved suspension in

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PBS was made. The optical density thereof, measured at 420 nm (O.D.420) must be 0.1. If necessary the concentrated bacterial mass is supplemented with PBS (pH 7.4) until the correct O.D. is obtained. An O.D.420 of 0.1 indicates the presence of 7×10^{11} bacteria per 30 ml, which is equivalent to 12 g wet weight of the bacterial mass.

For preparation of a crude mycobacterial extract 5 g wet weight of the bacterial mass was washed three times with PBS (pH 7.4). Centrifuging was then carried out at 3000 x g until the mass precipitated. The pellet was suspended in 50 ml PBS and stirred carefully to reduce formation of lumps to a minimum. To prevent lump forming 0.05% Tween 80 was optionally added. In order to avoid bacterial contamination 3 mg penicillin/streptomycin was added to this solution. The concentration was then brought with PBS to 2 g wet weight/ml.

The bacterial mass was subsequently broken open using an automatic French-X-press or RIBI press (American In-struments Company, Trevenollab. Inc. Maryland). The buckets were pre-cooled overnight at -20°C. Before use the buckets were held in a mixture of ethanol and dry ice (-20°C). After the buckets were filled with 1 g bacterial mass per bucket of 5 ml and cooled at -80°C for 20 minutes, the buckets were placed in the French-X press and 12 tons of pressure were applied by pushing in the plunger of the press. The buckets were then removed and cooled again at -80°C for 20 minutes. The buckets were inverted and treated for the second time. 10 tons of pressure were applied the second time. Cooling and breaking were then repeated a number of times, normally about 5 times. The disrupted cells were eluted with a suitable volume of PBS and subsequently centrifuged at 4°C at 300 x g for 10 minutes in order to remove the unbroken bacteria with the sediment. The collected supernatant was then centrifuged at 4°C and 145,000 x g for 2 hours. The pellet was suspended in 0.1 M Tris-HCl (pH 7.2), 0.01 M EDTA which contained 20 mM MgSO4.7H2O in a concentration of about 1 g per 10 ml. 1 mg RNase and 1 mg DNase were added per 10 ml volume. Incubation then took place overnight at 4°C with

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careful stirring. Incubation thereafter took place for 1
hour at 37°C and the lysate was centrifuged at 300 x g and
4°C for 10 minutes in order to remove the last-remaining
unbroken bacteria (this is further referred to as "starting
5 material").

2. Manufacture of membrane for assays.

A 12% polyacrylamide analytical gel of 1.5 mm thickness
was manufactured according to normal standard procedures. No
10 comb was used in the stacking gel. 5 mg of the starting
material obtained under 1. (100 mg per ml), respectively KP-
100 or SP-100 (see example 2) were used for each gel. 40
microlitres of this material was diluted with 1200
microlitres PBS. 300 microlitres 5x loading mixture (0.3 g
15 250 mM Tris-HCl, 1.0 g 10% SDS, 1.0 g 10% dithioerytreitol,
5 mg 0.05% bromophenol blue) was then added.

Incubation was carried out for 20 minutes at 65°C. 1500
microlitres were subsequently applied to the gel and
electrophoresis performed under the following conditions:
20 150 V for the run through the stacking gel for 30 minutes
and 100 V through the running gel for 6 hours.

In order to form a Western blot the proteins present in
the gel were transferred at 50 V for 3 hours to a
nitrocellulose membrane. After completion of the transfer
25 the membrane was coloured with 1.5% amido black for 2
minutes to check the membrane for irregularities and air
bubbles. The membrane was then decolourized in 0.05% Tween
80 in PBS with 1% BSA (bovine serum albumin). The membrane
was then cut into strips and was ready for use.
30

3. Immunodetection

The strips were incubated with 1:200 diluted human
serum. The serum was diluted in PBS with 3% BSA. The
incubation took place for 1 hour at room temperature. The
35 strips were subsequently washed three times (for 3 minutes
at a time) in PBS. The strips were then incubated with a
goat anti-total-human immunoglobulin-alkaline phosphatase
conjugate in a dilution of 1 to 1000 in PBS with 3% BSA and

0.05% Tween 80. Washing in PBS then took place again three times. The colour was developed with an NBT/BCIP (nitroblue tetrazolium/Bromo, Chloro Indolyl phosphate) colour solution (1 mg per 10 ml) to which 10 microlitres H_2O_2 were added.

5   The strips were incubated for a maximum of 2 hours in 1 ml of this solution per strip. The colour reaction was stopped by transferring the strips to 0.1 M Tris-HCl (pH 8.3), 0.01 M EDTA. The obtained patterns are interpreted by comparison with a reference pattern.

10   The results are shown in figures 1 and 2a-2c.

Figure 1 shows in the blots A and B an example of Western blotting patterns which are developed after incubation respectively with representative negative and positive sera (positive for bovine tuberculosis).

15   Blots C and D are an example of Western blotting patterns which are developed after incubation with a representative negative and positive serum sample, respectively (positive for Cattle Jones Disease).

Blots A and B: Lane 1: BCG crude extract, Lane 2: crude
20 extract of an M. tuberculosis strain, Lane 3: Myc. bovis crude extract.

Blots C and D: Lane 1: BCG derived KP-100, Lane 2: RIVM 7114 derived KP-100.

25 Interpretation of banding patterns from left to right is as follows.
Only the specific characteristics are stated herein.
Blot A: only the background bands can be observed in blots incubated with negative serum.
30 Blot B: region in the 10-16 KDa region in lane 3, 22 KDa band in lane 2, 31 KDa bands in lane 1 and 2, 14 KDa band in lane 2.
Blot C: only background bands can be observed in blots incubated with negative serum.
35 Blot D: 45-48 KDa doublet in lane 1 and 2, 22 and 25 KDa band in lane 1 and 2, 66 KDa band in lane 1 and 2, 27 KDa band in lane 1.

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Figure 2a is an example of different Western blotting patterns developed after incubation with representative variable sera of tuberculosis patients. Noticeable is the combination of different patterns demonstrating the presence

5 of different dominant bands, as shown in table 1. These band patterns function as "hallmarks" for TB patients as diagnosed serologically.

Applicable to all blots (from left to right): Lane 1 = BCG crude extract, Lane 2 = crude extract of an M.

10 tuberculosis strain.

Interpretation of banding patterns is as follows. Different blots (originating from different PAGE gels) are herein compared with each other.

15 Blot A: Mycobacterium avium infected patient.

Blot B-F: Tuberculosis patients.

Blot G: non-endemic negative serum.

Blot H: endemic negative serum (known recent contact, blot developed 2 weeks after patient returned to Netherlands from

20 endemic range).

Only "hallmarks" are mentioned.

Blot A: Mycobacterium avium infected patients, sera, band at 68 KDa in lane 1 and 2, range in 10-16 KDa in lane 1, band in the 58-60 KDa region in lane 2. Patient shows low IgA

25 titer in P-90 ELISA).

Blot B: 38-40 KDa band in lane 1 and 2, 10-16 KDa band in lane 1 and 2, band in 58-60 KDa region in lane 2, smear in 22-28 KDa region in lane 1.

Blot C: 16 KDa band in lane 1 and 2, bands in 58-60* KDa

30 region in lane 1 and 2, bands in 38-40 KDa region in lane 1 and 2, smear in 22-28 KDa region in lane 1, 33 KDa band in lane 1 and 2.

Blot D: 10 KDa band in 10-16 KDa region in lane 1, 16 KDa band in 10-16 KDa region in lane 2, 68 KDa band in lane 1

35 and 2, bands in 58-60* KDa region in lane 1 and 2.

Blot E: smear in 33-38 KDa region in lane 1 and 2, 16 KDa bands in lane 1 and 2, bands in 58-60* KDa region in lane 2.

Blot F: bands in 10-16, 22-28, 38-40, 58-60 regions and 68 KDa band in both lanes 1 and 2.

Blot G: non-endemic negative serum.

Blot H: endemic negative serum (known contact).

5 Figure 2b is an example of different Western blotting patterns developed after incubation with representative sera of patients with Lepromatous Leprosy (LL), Blot A and C, and Tuberculous Leprosy (TT), Blot B and D.

The "hallmark" patterns are shown in table 1 and are
10 for LL: distinctive 29/33 KDa doublet, and for TT: distinctive 64/65 KDa doublet (often observed as single band) or a very pronounced smear in the 30-64 KDa region.

To Blot A and B are applied: Lane 1: BCG crude extract, Lane 2: crude extract of a M. tuberculosis strain, Blot C:
15 Lane 1: Molecular marker, Lane 2: not relevant, Lane 3: BCG crude extract, Lane 4: crude extract of an M. tuberculosis strain, Blot D: Lane 1: BCG crude extract, Lane 2: crude extract of an M. tuberculosis strain, Lane 3: Molecular marker.

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Interpretation of banding patterns, wherein
only the "hallmarks" are mentioned, is as follows:

Blot A/C: 29-33 KDa doublet in lane 1 and 2.

Blot B/D: 64/65 KDa doublet in lane 1 and 2.

25 Noticeable is the very intensive smear in the 30-64 KDa range on blot D.

Finally, figure 2c is an example of different Western blotting patterns developed after incubation with representative sera of patients with Crohn's Disease. The
30 "hallmark" patterns are shown in table 3 and are for Crohn's Disease a pronounced 45/48 KDa doublet.

Applied to blot A is: BCG crude extract,

blot B: crude extract of an M. tuberculosis strain,

blot C: Mycobacterium avium crude extract,
35 blot D: molecular marker.

Interpretation of banding patterns is as follows:

All lanes show a distinctive colouring of the 45/48 KDa
doublet positivity, which indicates Crohn's Disease. The
45/48 KDa doublet reacts positively in 65% of all Crohn
5 patients.

EXAMPLE 2. Enzyme Immunoassay

1. Preparation of antigens

The starting material (see EXAMPLE 1 under 1.) was,
10 depending on the chosen M. tuberculosis strain, centrifuged
at 70,000 x g to 120,000 x g at 4°C for 2 hours. The pellet
was washed three times with PBS. Between the washing steps
centrifuging took place at 70,000 x g to 120,000 x g at 4°C
for 2 hours. The pellet was collected and resuspended in 10
15 ml PBS. The supernatant, SP-100, can also be used for
immunoblots (example 1) and enzyme immunoassays (this
example). The suspension was subsequently sonified for 2
minutes at 80 watts at 4°C. After the protein concentration
was determined, quantities of 100 µl were frozen at a
20 concentration of 1 mg/ml and stored at -80°C until time of
use (this preparation is designated with the term KP-100).

30 mg of the starting material was then applied in the
presence of loading mixture onto a preparatory 12%
polyacrylamide gel of 0.5 cm thickness after 20 minutes of
25 incubation at 65°C. Electrophoresis was carried out for 30
minutes at 150 V (stacking gel) and for 6 hours at 100 V
(running gel). The electrophoresis was stopped after the
blue colorant band had disappeared from the gel. The gel was
then cut into horizontal strips of 2 mm thickness which in
30 turn were divided into pieces of 1 cm length. The gel pieces
were each eluted overnight at 4°C in a tube with 5 ml
sterile distilled water. Thorough mixing thereafter took
place and the remaining gel pieces were centrifuged to the
bottom.

35 The elution was checked using a 12% polyacrylamide
analytical gel of 1.5 mm thickness. The gel was cast with a
comb. After 20 minutes incubation at 65°C 40 µl of each tube
with gel pieces was placed in the slots in the presence of

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10 µl 5x loading mixture. The electrophoresis was carried
out for 30 minutes at 150 V in the "stacking gel" and for 6
hours at 100 V in the "running gel". The electrophoresis was
stopped and the gel made ready for preparation of a Western
5   blot. The blotting procedure is described in EXAMPLE 1 under
2. Similar results were obtained using HPLC, FPLC and other
routine separating procedures.

    In order to establish which fractions contain the
relevant antigens, strips of the blot were incubated with
10 sera of patients with lepromatous leprosy, tuberculous
leprosy and Crohn's Disease. Shown herewith are respectively
the 29/33 KDa antigens, the 64/65 KDa antigen and the 45/48
KDa antigens. The complex formation was visualized using
anti-human IgG peroxidase conjugate and DAB. The desired
15 fractions were collected, combined and used to coat a
microtiter plate (see below).

2. EIA.

    Microtiter plates are coated (via standard techniques)
20 with either KP-100, SP-100, starting material, whole
bacteria, 29/33 KDa, 64/65 KDa or 45/48 KDa antigens.

    After coating the plates are blocked, in order to
prevent an aspecific binding of serum components, with a 3%
bovine serum albumin (BSA) solution. Plates are then dried
25 and stored at 4°C.

2.1. Tuberculosis EIA test (microtiter plates coated with
KP-100)

    Test sera are pipetted in a 1:100 dilution into the
30 coated wells of a microtiter plate. The reaction takes place
for 1 hour at 37°C. Aspecific serum components and non-
binding serum components are washed away in a washing step.
A second incubation with a suitable dilution of an anti-
human IgA peroxidase conjugate is carried out again for 1
35 hour at 37°C, and excess conjugate is then washed away.

    Indication of human antibodies of the sub-type IgA
binding specifically to KP-100 takes place by adding TMB
(tetramethylbenzidine) to the wells.

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Binding enzyme results in the occurrence of a blue
colour which, after addition of a colouring stop solution,
changes to yellow. This yellow colour has an absorption
maximum of 450 nm.

5   The intensity of the resulting colour is proportional
to the amount of bound KP-100-specific IgA.

The results are shown in the tables below.

In the described test patient and control sera are used
from two different populations.

10 A = Endemic area (Africa, Ghana)

B = Non-endemic area (Europe, the Netherlands).

Each population is sub-divided into 4 sub-groups,
namely:

Group 1 = culture confirmed TB patients

15 Group 2 = negative control group (normal healthy individuals

Group 3 = suspected positives (TB contacts)

Group 4 = suspected negatives (no known data, but certainly
            no TB, possibly leprosy or other aspecific
            Mycobacteriosis).

20   The test is performed with two kits having different
lot numbers and production dates.

Interpretation of the test results takes place on the
basis of the so-called calibration line which is made up of
control sera with a determined arbitrary unit definition

25 which corresponds to a known OD value (1 unit, 4 units and 8
units).

Each time a test is carried out the units are included
in the assay. Found sample values can then be related to the
unit definition.

30   A test serum can be considered positive when the result
found in the test scores higher than 2.1 units.

A test serum can be considered negative when the result
found scores lower than 1.2 units.

Test sera with unit values between 2.1 and 1.2 units

35 fall into the set so-called reconfirmation zone. This means
that in the first instance positivity or negativity for
tuberculosis cannot be determined with this test.

Reconfirmation of these sera takes place using the described Western blot strips with which, after serum incubation on the basis of banding patterns and specific "hallmarks", an answer can be given to the question of
5 whether the test serum is positive (bands present) or negative (bands absent).

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Table 3:

Population A. Endemic range:

Group 1:

SERUM NO.	# UNITS	TEST SCORE	REMARKS
1	2.44	positive	culture positive
2	3.43	positive	culture positive
3	1.78	reconfirmation	culture positive
4	1.37	reconfirmation	culture positive
5	5.74	positive	culture positive
6	3.21	positive	culture positive
7	1.66	reconfirmation	culture positive
8	2.00	reconfirmation	culture positive
9	2.00	reconfirmation	culture positive

Table 4:

Population A. Group 2:

SERUM NO.	# UNITS	TEST SCORE	REMARKS
10	1.16	negative	healthy individual
11	0,86	negative	healthy individual
12	0.77	negative	healthy individual
13	0.64	negative	healthy individual
14	0.74	negative	healthy individual
15	0.79	negative	healthy individual

Table 5:

Population A. Group 3:

SERUM NO.	# UNITS	TEST SCORE	REMARKS
16	3.13	positive	sick individual
17	1.57	reconfirmation	sick individual
18	1.59	reconfirmation	sick individual
19	5.39	positive	sick individual
20	1.97	reconfirmation	sick individual
21	2.29	positive	sick individual
22	0.48	negative	sick individual

Table 6:

Population A. Group 4:

SERUM NO.	# UNITS	TEST SCORE	REMARKS
23	1.54	reconfirmation	normal control
24	1.76	reconfirmation	normal control
25	0.58	negative	culture negative
26	1.03	negative	culture negative
27	0.79	negative	culture negative
28	0.89	negative	culture negative

Table 7:

Population B. Non-Endemic range:

Group 1:

SERUM NO.	# UNITS	TEST SCORE	REMARKS
1	2.24	positive	culture positive
2	2.53	positive	culture positive
3	4.40	positive	culture positive
4	14.95	positive	culture positive
5	16.82	positive	culture positive
6	10.54	positive	culture positive
7	5.70	positive	culture positive
8	6.72	positive	culture positive
9	5.06	positive	culture positive

Table 8:

Population B.

Group 2:

SERUM NO.	# UNITS	TEST SCORE	REMARKS
10	1.04	negative	healthy individual
11	0.92	negative	healthy individual
12	0.85	negative	healthy individual
13	0.20	negative	healthy individual
14	0.42	negative	healthy individual
15	0.90	negative	healthy individual
16	0.35	negative	healthy individual
17	0.73	negative	healthy individual

Table 9:

Population B.

Group 3:

SERUM NO.	# UNITS	TEST SCORE	REMARKS
18	2.23	positive	sick individual
19	4.70	positive	sick individual
20	1.22	reconfirmation	sick individual
21	1.43	reconfirmation	sick individual
22	2.21	positive	sick individual with TB history
23	6.38	positive	sick individual
24	1.59	reconfirmation	sick individual

Table 10:

Population B.

Group 4:

SERUM NO.	# UNITS	TEST SCORE	REMARKS
25	0.20	negative	patient resistant to drug therapy

1. Method for identifying a <u>Mycobacterium</u> species responsible for a mycobacterial infection in human or animal, comprising the steps of:

a) selecting a suitable mycobacterial species and strain;

b) preparing at least one mycobacterial antigen, respectively antigen preparation, comprising at least one selected immuno-cross-reactive antigen component;

c) binding the antigen, respectively the antigen preparation to a suitable carrier;

d) causing the bound immuno-cross-reactive antigen component(s) to react with antibodies from serum of an individual infected with a <u>Mycobacterium</u> species;

e) making visible antigen-antibody reactions for a suitable antibody (sub-)class; and

f) identifying the responsible <u>Mycobacterium</u> species on the basis of the reactions which are made visible.

13. Diagnostic kit comprising a dip-stick on which is arranged a carrier strip with mycobacterial antigens, comprising at least one selected immuno-cross-reactive antigen component, bound thereto, and means for visualizing antigen-antibody reactions occurring on the carrier after contact with the serum for testing.

15. Diagnostic kit comprising an immunoblot with mycobacterial antigens, comprising at least one selected immuno-cross-reactive antigen component, which antigens are separated by electrophoresis, binding thereto, and means for visualizing antigen-antibody reactions occurring on the immunoblot after contact with the serum for testing.

CLAIMS

1. Method for identifying a <u>Mycobacterium</u> species responsible for a mycobacterial infection in human or animal, comprising the steps of:

a) selecting a suitable mycobacterial species and
5   strain;

b) preparing at least one mycobacterial antigen, respectively antigen preparation;

c) binding the antigen, respectively the antigen preparation to a suitable carrier;

10   d) causing the bound antigen to react with antibodies from serum of an individual infected with a <u>Mycobacterium</u> species;

e) making visible antigen-antibody reactions for a suitable antibody (sub-)class; and

15   f) identifying the responsible <u>Mycobacterium</u> species on the basis of the reactions which are made visible.

2. Method as claimed in claim 1, **characterized in that** the antigen preparation is separated by electrophoresis prior to step c) and the carrier is a membrane to which the
20   antigen is bound by means of electroblotting.

3. Method as claimed in claim 2, **characterized in that** the membrane is a nitrocellulose membrane.

4. Method as claimed in claim 1, **characterized in that** the <u>Mycobacterium</u> species responsible for the mycobacterial
25   infection is identified on the basis of a reference pattern.

5. Method as claimed in any of the claims 1-4, **characterized in that** the antigen preparation is a total protein preparation of Mycobacterium.

6. Method as claimed in any of the claims 1-4, **char-**
30   **acterized in that** the antigen preparation is a KP-100 or SP-100 fraction of the total protein preparation.

7. Method as claimed in claim 1, **characterized in that** the antigen is specific for one <u>Mycobacterium</u> species and the carrier is a microtiter plate.

35   8. Method as claimed in claim 7, **characterized in that** the specific antigen is chosen from the group consisting of

mycobacterial immuno-cross-reactive antigen components with a molecular weight of respectively 29/33 KDa, 45/48 KDa, 64 KDa and the antigen fractions KP-100 or SP-100 or combinations thereof.

5   9. Method as claimed in claim 7 or 8, **characterized in** **that** the antigen-antibody reactions are made visible using antibody-enzyme conjugates directed against antibodies of an isotype chosen from IgG, IgM, IgA.

10. Method as claimed in claim 9, **characterized in that** 10   the enzyme is peroxidase.

11. Method as claimed in claim 1, **characterized in that** the carrier is a dip-stick with mycobacterial antigens binding thereto, that the antigen is brought into reaction with antibodies from serum for testing by dipping the stick 15   in a serum monster for testing, and that the antigen-antibody reactions are made visible by subsequently dipping the stick in a solution with anti-antibody antibody-enzyme conjugate and a colour substrate for the relevant enzyme.

12. Method as claimed in claim 1, **characterized in that** 20   the antigen is bound to the wells of a microtiter plate, that the antigen is brought into reaction with antibodies from the serum to be tested by placing in the wells a sample of the serum to be tested, that the non-binding antibody is removed, and that the antigen-antibody reaction is made 25   visible by subsequently placing in the wells an anti-antibody antibody-enzyme conjugate and a colour substrate for the relevant enzyme.

13. Diagnostic kit comprising a dip-stick on which is arranged a carrier strip with mycobacterial antigens bound 30   thereto, and means for visualizing antigen-antibody reactions occurring on the carrier after contact with the serum for testing.

14. Diagnostic kit comprising a microtiter plate in the wells of which a specified antibody is arranged, and means 35   for visualizing antigen-antibody reactions occurring in the wells after contact with the serum for testing.

15. Diagnostic kit comprising an immunoblot with mycobacterial antigens separated by electrophoresis binding

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